# *Agrobacterium tumefaciens*-mediated transformation of *Colletotrichum graminicola* and *Colletotrichum sublineolum*

**Flowers and Vaillancourt, 2005. Current Genetics 48: 380-388**

NOTE added by L. Vaillancourt: The advantage of this method is that we can use spores rather than protoplasts for transformation. This is the only method that we have been able to use to transform C. sublineolum. The disadvantage of the method seems to be that we often get silencing of the phenotypes, particularly of the fluorescent phenotypes, especially after long storage. We don't really understand why but we have not had this problem with our PEG transformants, even though we use the same genes and promoters to transform them.

## Making competent cells of *Agrobacterium tumefaciens*

Inoculate 5 ml LB broth amended with Rif 100/Tet 5* with a colony of *A. tumefaciens* or 15 µl of *A tumefaciens* glycerol stock. We have used two strains, AGL-1 and C58C1. AGL-1 works much better.

Incubate overnight at 28°C shaking 250 RPM.

Next day inoculate 50 ml LB broth amended with Rif 100/Tet 5 in a sterile 250 ml flask with 2 ml of the above overnight culture. Make glycerol stock of the remainder (850 µl of culture and 150 µl of sterile glycerol).

Incubate flask at 28°C shaking 250 RPM for ~8 hours to OD 600 nm of 0.6.

Chill the flask on ice for 5 minutes.
Pellet cells in 50 ml Falcon tubes at 3000 RPM at 4°C for 5 min.
Resuspend cells in 1 ml of 20 mM $CaCl_2$

Make 100 µl aliquots and store at –80°C (use 50 µl of cells per transformation)

***Antibiotic Stock Solutions**
These antibiotics are used to reduce bacterial contamination since *A. tumefaciens* grows slowly. However, the AGL-1 strain does not grow with these antibiotics, so they should not be used if strain AGL-1 is being used.

Rifamipicin 25 mg/ml in 100% methanol (add 4 ml/L LB = Rif 100)
Tetracycline 5 mg/ml in ethanol (add 1 ml/L LB = Tet 5

Store the antibiotic solutions at –20°C

Add all antibiotics after autoclaving LB

**Transformation of *A. tumefaciens***

Add 10 μl of plasmid miniprep DNA to 50 μl of ***A. tumefaciens*** competent cells in a 1.5 ml Eppendorf tube.

Mix gently by tapping tube.

Freeze tubes in liquid nitrogen. Wait until bubbling subsides then remove the tube.

Thaw frozen tubes in 37°C water bath for 5 minutes.

Add 0.5 ml LB Broth without antibiotics.

Incubate cells at 28°C/250 RPM/2-3 hours

Plate the entire contents on LB Rif 100/Tet* 5/Kan 50 plates (or pellet cells and resuspend with 150 ul LB broth before plating)

Incubate plates at 28°C/2days

Pick one transformant colony and inoculate 5 ml LB Broth Rif 100/Tet 5/Kan 50.

Incubate overnight 28°C/250 RPM

Make glycerol stock of transformed cells. (850 μl of cells + 150 μl sterile glycerol)

Streak LB agar amended with Kan 50 with glycerol stock and incubate at 28°C/48 hours before use for fungal transformation.

***Antibiotic Stock Solutions**
Rifampicin and Tetracycline are used to reduce bacterial contamination since *A. tumefaciens* grows slowly. However, the AGL-1 strain does not grow with these antibiotics, so they should not be used if strain AGL-1 is being used.

Rifamipicin 25 mg/ml in 100% methanol (add 4 ml/L LB = Rif 100)
Tetracycline 5 mg/ml in ethanol (add 1 ml/L LB = Tet 5

Kanamycin 25 mg/ml in water (add 2 ml/L LB = Kan 50) Kanamycin is the selection antibiotic for transformed fungal colonies provided that the plasmid has the gene for Kanamycin resistance. You must always use kanamycin, therefore.

Store all antibiotics at –20 C

Add all antibiotics to sterile cooled media before pouring plates or inoculating broth.

## *Agobacterium*-mediated transformation of *C. graminicola*

Streak out the *Agrobacterium* (Agro) containing plasmid onto LB agar augmented with 500 μl of a 50mg/ml stock of Kanamycin /500 mls media which gives us a concentration of 50 μg/ml of Kanamycin (Kan 50). Incubate upside down at 29°C for 48 h. The plasmid-*Agrobacterium* line we use is pRAN-AgL-1. This is strain AGL-1 transformed with the plasmid pBin-GFP-hph.

Using a long sterile loop, transfer an actively growing colony of Agro to 5 mls of Minimal Media Broth augmented with 5 μl thiamin (1 g/ml stock) and 5 μl of Kan 50 stock. Incubate 29°C/230-250 rpm/48 hours.

Transfer 150-200 μl of the Minimal Media Broth Culture to 5 mls of liquid Induction Media (IM) augmented with 10 μl acetosyringone (AS, 100 mM stock) and 5 μl thiamine (1 g/ml stock). Incubate 29°C/230-250 rpm/about 6 hours until the OD600 reading is at least 0.25

Co-cultivate 300 μl of the Agro IM culture and 100 μl of fungal spores (harvested from an actively growing plate, pelleted, washed 2 x, concentration adjusted to $1x10^6$ spores/ml) in a 1.5 ml micro centrifuge tube. Allow the tube to sit on the lab bench while you are placing small pieces of nitrocellulose membrane filters (Millipore 0.45 um HA) on the solid IM media augmented with 200 μl of a 100 μM stock of AS and 100 μl of Thiamine (1 g/ml). This takes about 20-25 minutes.

Drop 200-400 μl of the co-cultivated Agro/spore solution on the nitrocellulose pieces. Spread the solution across the pieces with a sterile glass rod or a sterile plastic spreader or sterile blue micropestile. Incubate the plates either on the lab bench or in the usual growing area for the fungus you are transforming. Incubate for 2-5 days. Control for this experiment is 5 pieces of nitrocellulose on augmented IM media inoculated with spore suspension only. Incubate as listed above.

Use sterile forceps to transfer about 12 nitrocellulose pieces onto PDA augmented with
-100 μl of Cefotaxime (stock solution 200 mg/ml) added to each 100 mls of media
  Concentration of Cefotaxime in 100 mls of PDA is 200 μg/ml.
-100 μl of Carbenicillin (stock solution 250 mg/ml) added to each 100 mls of media
  Concentration of Carbenicillin in 100 mls of PDA is 250 μg/ml.
-63 μl of Hygromycin B in 100 mls of PDA. (This is based on the concentration of Hygromycin B being 394 mg/ml.)
  Concentration of Hygromycin B in 100 mls of PDA is 250 μg/ml
Incubate the PDA plates either at room temp or in the required growing conditions for the fungus.

Transfer transformant colonies to PDA augmented with Hygromycin B 250 μg/ml.

**MEDIA RECIPES**

**Minimal Media 50X Stock**
Dissolve one at a time in this order
-150 g Na3Citrate (5 1/2 $H_2O$) in 750 ml double distilled or Nan-O-Pure water or can use 125 g Na3 Citrate ($2H_2O$) in 775 ml double distilled or Nan-O-Pure water
-250 g $KH_2PO_4$, anhydrous
-100 g $NH_4NO_3$, anhydrous
-10 g $MgSO_4$ ($7H_2O$)
-*5 g $CaCl_2$ ($2H_2O$) dissolved separately in 20 ml double distilled or Nan-O-Pure water and solution added slowly
-2.5 ml Biotin Stock Solution (dissolve 5 mg in 100 ml 50% ethanol; store –20 C)
-5 ml of **Trace Element Solution**

**Trace Element Solution**
Dissolve in 95 ml double distilled or Nan-O-Pure water:
-5 g citric acid ($1H_2O$);
-5 g $ZnSo_4$ ($7H_2O$);
-1 g $Fe(NH_4)_2(SO_4)_2$ ($6H_2O$);
-0.25g $CuSO_4$ ($5H_2O$);
-0.05 g $MnSO_4$ (1 $H_2O$);
-0.05 g $H_3BO_3$;
-0.05 g $Na_2MoO_4$ ($2H_2O$).

-Add 5 ml Chloroform as a preservative to the MM 50X Stock **(DO NOT AUTOCLAVE**) and store solution at room temperature. Chloroform stays on the bottom. Do not disturb this layer.

For Minimal Media (MM) made a 1X solution of the above stock and add 1% Sucrose then. aliquot 5ml into glass test tubes. **AUTOCLAVE.** Immediately prior to use add 5 µl thiamine and 5 µl of Kanamycin (50 mg/ml Stock)
After autoclaving the media can remain at room temperature.
1 ml of 50X Minimal Media Stock + 49 mls sterile Nan-O-Pure Water + 0.5 g sucrose
Remember to add the thiamine and Kanamycin right before inoculation of the MM.

For Induction Media per 50 mls: 10 tubes at 5mls each
2 ml Minimal Media 50X Stock
2 ml 1 M MES (pH 5.3) previously autoclaved
0.5 ml 1 M Glucose previously autoclaved
0.25 ml 100% Glycerol previously autoclaved
Adjust volume to 50 mls with sterile Nan-O-Pure water
Autoclave and Cool. Store at room temperature until needed.
Add 10 ul AS (100 mM stock) and 5 µl thiamine (1 g/L stock) to each tube right before use.

For Solid Induction Media per 100 mls
2 ml Minimal Media 50X Stock
4 ml 1 M MES (pH 5.3) (final concentration is 40 mM)
0.5 ml 1 M Glucose (final concentration is 5 mM)
0.5 ml 100% Glycerol (final concentration is 0.5%)
Adjust volulme to 100 mls then add
1.5g agar
Autoclave and cool in 50-55 c water bath. Add 200 μl AS stock and 100 μl thiamine stock before pouring plates.

Stocks for vitamins and antibiotics: Add any of the antibiotics and vitamins to cooled media prior to pouring agar or inoculating broth.

Thiamine 1 g/ml Stock
Dissolve 1 g /1 ml sterile Nan-O-Pure water,
Filter sterilize and store in the –20 freezer
Use 5 μl/5 ml of minimal media
Use 100 μl/ 100 mls of solid induction media.

Kanamycin 50mg/ml Stock
Dissolve 50 mg/ml of sterile Nan-O-Pure water
Filter sterilize and store in the –20 freezer
Use 5 μl/5 ml; 500 μl/500 mls (Concentration of 50 μg/ml)

Acetosyringone (AS) 100 mM Stock
Acetosyringone 196.20 gfw.
Dissolve in 95% ethanol and adjust volume with water
Dissolve 0.3924g in 12 mls 95% ethanol then add 8 mls of sterile Nan-O-Pure water to equal 20 mls.
Filter sterilize store in –20 C
Use 5 μl in liquid IM; Use 200 μl in solid IM

Cefotaxime 200 mg/ml Stock
Dissolve 200 mg Cefotaxime in 1 ml of sterile Nan-O-Pure water.
Filter Sterilize. Store in –20 C.
Add 100 μl Cefotaxime stock /100 mls media (Concentration 200 μg/ml)

Carbenicillin 250 mg/ml Stock
Dissolve 250 mg Carbenicillin in 1 ml sterile Nan-O-Pure water.
Filter Sterilize. Store in –20 C.
Add 100 μl of Carbenicillin stock /100 mls media (Concentration 250 μg/ml)

Hygromycin B Stock

Our Hygromycin B is a liquid bought at the concentration of 394 mg/ml. Using the formula C1V1 = C2V2 gives the amount of Hygromycin B to add to the known volume of media to equal the desired concentration. In this instance add 63 μl of Hygromycin B stock to 100 mls media to obtain the final concentration of 250 μg/ml